

Antiangiogenic and Antitumor Effects of Bevacizumab in Patients With Inflammatory and Locally Advanced Breast Cancer

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ABSTRACT

Purpose

Vascular endothelial growth factor (VEGF) is a potent molecule that mediates tumor angiogenesis primarily through VEGF receptor 2 (VEGFR2). Bevacizumab, a recombinant humanized monoclonal antibody to VEGF, was administered to previously untreated patients to evaluate parameters of angiogenesis.

Patients and Methods

Twenty-one patients with inflammatory and locally advanced breast cancer were treated with bevacizumab for cycle 1 (15 mg/kg on day 1) followed by six cycles of bevacizumab with doxorubicin (50 mg/m²) and docetaxel (75 mg/m²) every 3 weeks. After locoregional therapy, patients received eight cycles of bevacizumab alone, and hormonal therapy when indicated. Tumor biopsies and dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) were obtained at baseline, and after cycles 1, 4, and 7.

Results

A median decrease of 66.7% in phosphorylated VEGFR2 (Y951) in tumor cells ($P = .004$) and median increase of 128.9% in tumor apoptosis ($P = .0008$) were seen after bevacizumab alone. These changes persisted with the addition of chemotherapy. There were no significant changes in microvessel density or VEGF-A expression. On DCE-MRI, parameters reflecting reduced angiogenesis, a median decrease of 34.4% in the inflow transfer rate constant ($P = .003$), 15.0% in the backflow extravascular-extracellular rate constant ($P = .0007$) and 14.3% in extravascular-extracellular volume fraction ($P = .002$) were seen after bevacizumab alone.

Conclusion

Bevacizumab has inhibitory effects on VEGF receptor activation and vascular permeability, and induces apoptosis in tumor cells.

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INTRODUCTION

Inflammatory breast cancer (IBC) is a highly aggressive subtype of locally advanced breast cancer representing approximately 5% of all breast cancers.^{1,2} It is clinically characterized by skin erythema and edema known as *peau d'orange*, and molecularly characterized by a high vascularity with an increased microvessel density (MVD).³ IBC has high expression levels of angiogenic factors such as **vascular endothelial growth factor (VEGF)** and basic fibroblast growth factor.⁴

VEGF is one of the most potent promoters of **angiogenesis** involved in endothelial cell growth and motility and blood vessel permeability.⁵ Over-

expression of VEGF has been observed in a variety of cancers including breast cancer, and is associated with a worse relapse-free and overall survival (OS) compared with nonoverexpressing cancers.⁶⁻⁸ Most functions of VEGF are mediated through the tyrosine kinase receptor VEGFR2 (also known as KDR or Flk-1).⁹

Bevacizumab is a humanized monoclonal antibody to VEGF (Avastin; Genentech Inc, South San Francisco, CA) that recognizes all isoforms of VEGF-A. Preclinical models have shown regression of solid tumor growth and angiogenesis with anti-VEGF monoclonal antibodies alone or in combination with chemotherapy.¹⁰⁻¹²

A pilot study was conducted in patients with previously untreated IBC or locally advanced breast

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Terms in **blue** are defined in the glossary, found at the end of this article and online at www.jco.org.

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Table 1. Experimental Conditions Used in Immunohistochemistry

Antibody	Manufacturer	Dilution	Positive Control Tissue	Antigen Retrieval
KDR (55B11), rabbit monoclonal	Cell Signaling Technology (Beverly, MA)	1:100	Healthy kidney	DAKO buffer (pH, 10.0), MW until boiling, then, at 150 W for 10 minutes
p-KDR (Y996), rabbit polyclonal	Cell Signaling Technology (Beverly, MA)	1:100	Breast cancer specimen	DAKO buffer (pH, 10.0), MW until boiling, then, at 150 W for 10 minutes
p-KDR (Y951), rabbit polyclonal	Santa Cruz Inc (Santa Cruz, CA)	1:150	Breast cancer specimen	DAKO buffer (pH, 10.0), MW until boiling, then, at 150 W for 10 minutes
Ki67 (MIB-1), mouse monoclonal	DAKO Corp (Carpinteria, CA)	1:50	Healthy tonsil	DAKO buffer, pH6.0, MW until boiling, then, at 150 W for 10 minutes
CD31 (JC70A), mouse monoclonal	DAKO Corp (Carpinteria, CA)	1:20	Breast cancer specimen	DAKO buffer (pH, 6.0), MW until boiling, then at 150 W for 5 minutes
VEGF A (JH121), mouse monoclonal	AngioBio Corp (Del Mar, CA)	1:50	Colon cancer specimen	DAKO buffer (pH,6.0), WB at 95°C for 20 minutes

Abbreviations: MW, microwave; WB, water bath.

cancer (LABC) with bevacizumab alone and in combination with chemotherapy. The objectives were to access molecular changes with bevacizumab alone and in combination with chemotherapy. Those included tissue VEGF, activated VEGFR2 status (phosphorylated VEGFR2 [p-VEGFR2]), total VEGFR2, tumor MVD, tumor cell apoptosis, and proliferation. Also, vascular permeability was accessed by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI).

PATIENTS AND METHODS

Study Design and Clinical Evaluation

This pilot study was approved by the institutional review board of the National Cancer Institute (NCI; National Institutes of Health, Bethesda, MD). All patients signed informed consent before enrollment. The trial was designed to administer bevacizumab in cycle 1 and in combination with doxorubicin and docetaxel for cycle (C-) 2 through C7 in patients with previously untreated

IBC and LABC. Serial investigations with DCE-MRI and tumor biopsies were performed at baseline, and after C1, C4, and C7. Tissue from surgery was used as the C7 specimen if surgery was performed.

Patients were evaluated every 3 weeks. Cardiac function was evaluated with an ECG and echocardiogram or multigated acquisition (MUGA) at baseline and after C4, C7, surgery (before C8), C11, and C15. Radiographic tumor assessments were made at baseline and after C1, C4, and C7 and were reported using the Response Criteria in Solid Tumors (RECIST).¹³ Toxicities were assessed using the NCI Common Toxicity Criteria version 2.0.¹⁴

Patient Eligibility

Patient eligibility criteria included previously untreated stage III or IV IBC or LABC, age \geq 18 years, Eastern Cooperative Oncology Group (ECOG) performance status 0 to 2, left ventricular ejection fraction (LVEF) \geq 50% without clinical symptoms or signs of heart failure, and adequate organ function. IBC was defined as histologically proven invasive adenocarcinoma with clinical signs of diffuse erythema and edema involving more than half of the breast with or without an underlying tumor mass. LABC was defined as stage IIB, IIIA, IIIB or IIIC breast cancer utilizing 2002 American Joint Committee on Cancer (AJCC) staging guidelines.¹⁵

Table 2. Baseline Patient Characteristics

Characteristic	No. of Patients
Enrolled patients	21
Age, years	
Median	50
Range	35-73
Stage	
III	17
IV	4
Histology	
Ductal	19
Lobular	1
Mixed ductal and lobular	1
Grade	
2	9
3	12
Estrogen-receptor positive	9
Progesterone-receptor positive	6
Her2/neu positive	
IHC (3+)	2
FISH positive	2
Positive skin biopsy	11

Abbreviations: IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

Table 3. Selected Toxicities for Patients Receiving Therapy*

Toxicity	Common Toxicity Criteria Grade (No.)			
	1	2	3	4
Bleeding	6	0	0	0
Epistaxis	15	0	0	0
Headache	5	0	1	0
Hypertension	0	2	8	0
LVEF dysfunction	0	2	0	0
Proteinuria	1	1	0	0
Thrombosis	0	0	1	0
Wound healing	5	0	0	0
Diarrhea	5	2	4	0
Fatigue	13	4	4	0
Febrile neutropenia	0	0	4	0
Neutropenia	0	2	3	15
Rhinitis	9	8	0	0
Sensory neuropathy	10	2	0	0
Thrombocytopenia	15	2	2	0

Abbreviation: LVEF, left ventricular ejection fraction.

*Listed grade of toxicity is the worst grade for patient while on study.

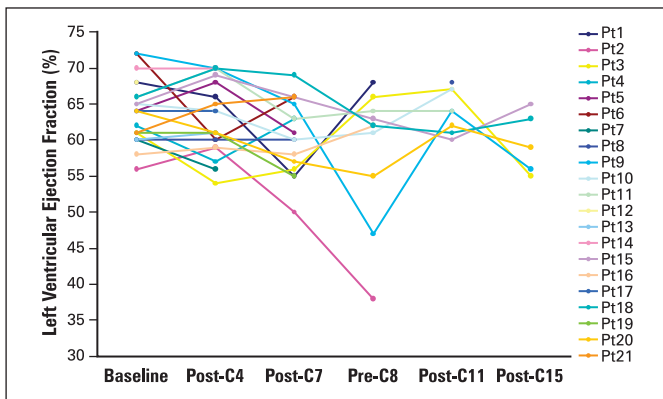


Fig 1. Absolute change in left ventricular ejection fraction of patients while on study. Pt, patient; C-, cycle.

Drug Administration

Bevacizumab at 15 mg/kg on day 1 was administered for C1. C2 to C7 consisted of doxorubicin at 50 mg/m² followed by docetaxel at 75 mg/m² on day 1 every 3 weeks, in addition to bevacizumab. Dexamethasone 8 mg was administered orally every 12 hours for 3 days starting 24 hours before docetaxel administration. Granulocyte colony-stimulating factors (G-CSFs) were administered on day 2 for C2-C7. Patients had surgery at a minimum of 4 weeks after C7 of bevacizumab and chemotherapy. Radiation therapy to the chest wall and supraclavicular area was begun after complete recovery from surgery. Eight additional cycles of bevacizumab (15 mg/kg every 3 weeks) were administered, for a total of 15 cycles. Patients with hormone-positive tumors received tamoxifen or an aromatase inhibitor.

Tissue Correlative Studies

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a standard avidin-biotin-peroxidase complex indirect immunoperoxidase procedure as described previously.¹⁶ Tumor content was evaluated on sections stained with hematoxylin and eosin (M.S. and S.Y.). Antibodies and immunohistochemistry (IHC) conditions are described in Table 1.

Dual labeling for Ki67 and CD31 was carried out to detect proliferative endothelial cells using the EnVision Doublestain System (DakoCytomation, Carpinteria, CA). Staining was first carried out for Ki67 as described in the preceding paragraph then, after blocking an antibody to CD31 (1:20 dilution), was incubated at 4°C overnight and reaction was revealed with permanent red. CD31-positive cells were visualized as red cytoplasmic staining, and Ki-67 cells were visualized as brown nuclear staining.

The dual Ki67/CD31 staining was scored manually by two investigators (H.K. and S.H.) without knowledge of any clinical data. The specimen was considered inadequate if the IHC stain for CD31 failed to outline vascular channels. CD31-positive cells, up to a maximum of 200, within regions of tumor and normal epithelium extending to one 20× objective diameter from

the edge of epithelium were enumerated. Dual positive cells were counted within the same area using previously described methods,³ resulting in a ratio of Ki67+:CD31+/CD31+ cells. If there were any vessels present in the biopsy by morphology but not revealed by CD31, the biopsy was considered inadequate for CD31 staining.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (R&D Systems, Minneapolis, MN) measures the fragmented DNA in apoptotic cells. DNA breaks at 3'-DNA ends were labeled with biotinylated nucleotides catalyzed by TdT. An avidin-conjugated horseradish peroxidase specifically binds to the biotinylated DNA fragments (Vector Laboratories Inc, Burlingame, CA) and generates a brown precipitate in the presence of diaminobenzidine. Nuclease-treated breast cancer specimen section was used as a positive control, and the same tumor section without nuclease treatment was used as a negative control.

Immunohistochemical staining signal was analyzed quantitatively with the assistance of the Automated Cellular Imaging System (ACIS; Chroma-Vision Medical Systems Inc, San Juan Capistrano, CA) using the method described previously.^{16,17} Images from the ACIS instrument were reviewed by two investigators (S.Y. and S.H.) without knowledge of any clinical data. To compare expression levels of markers before and after treatment objectively, a hot spot feature of the ACIS was used to identify sites of tumor staining where six areas of tumor were scored with an ×40 tool or a free-scoring tool. For Ki67 and TUNEL assay, the instrument calculated an average labeling percentage for tumor cells. An index of staining was calculated for VEGFR2, p-VEGFR2, and VEGF-A. The staining index is expressed as the percentage of staining multiplied by staining intensity after subtracting the tissue readouts of the corresponding negative control for each marker per 100. For the determination of mean MVD, slides were scanned by ACIS at low power (×10). Six areas of vessel-containing hot spots were identified on the images and, then, those selected were rescanned by ACIS at a high power (×40). The computer software enumerated the number of CD31-positive vessels and converted to the number of vessels per mm².

DCE-MRI

DCE-MRI was performed on a 1.5 T GE system (GE Healthcare, Waukesha, WI). Three sets of baseline images were obtained before intravenous contrast administration (Magnevist, gadolinium dimeglumine pentastate; Berlex Laboratories, Wayne, NJ). The images were analyzed using an IDL-based analysis program (IDL Corp, Boulder, CO) and a modified version of Cine Tools (GE Healthcare, Waukesha, WI).¹⁸ Regions of interest (ROIs) were drawn with an automated drawing tool in the software supplied with the general kinetic model (GKM) around tumor areas. Using a two compartment model, the following parameters were obtained: K^{trans} (forward transfer constant from vascular space to the tumor), k_{ep} (reverse rate constant from tumor to the vascular space), and v_e (extravascular volume fraction).

Relative Dose Intensity

Actual dose-intensity (ADI) indicated how much individual agent was administered per week. Relative dose-intensity was calculated by dividing the ADI by the planned dose-intensity (planned total dose/No. of weeks).

Table 4. Parameters of Dynamic Contrast-Enhanced Magnetic Resonance Imaging

	BL to C1 (n = 18)		BL to C4 (n = 19)		BL to C7 (n = 15)		C1 to C4 (n = 17)		C4 to C7 (n = 15)	
	%	P	%	P	%	P	%	P	%	P
K^{trans}	-34.4	.003	-58.0	< .0001	-75.5	.0001	-57.5	.011	-12.4	.76
k_{ep}	-15.0	.0007	-49.4	.002	-59.1	.0001	-46.8	.045	-14.2	.89
v_e	-14.3	.002	-20.4	.020	-20.2	.095	-4.4	.58	-11.3	.56

NOTE. All percentage values represent median relative changes for the later time point compared to the earlier one indicated. P values are two tailed and were calculated using the Wilcoxon signed rank test.

Abbreviations: BL, baseline; C-, cycle; K^{trans} , forward transfer constant from vascular space to the tumor; k_{ep} , reverse rate constant from tumor to the vascular space; v_e , extravascular volume fraction.

Statistical Analyses

This study was designed as a single-arm, single-stage pilot study with 20 assessable subjects. This number was selected to permit the study to have 95% power to be able to detect a significant change from baseline to the end of C1 in each of up to four parameters that was equal to one standard deviation of the change, using a two-sided .05 α level Wilcoxon signed rank test (or paired t test if appropriate).

For each parameter, it was determined that the relative change from baseline (expressed as a percentage) was less dependent on the baseline value than was the difference. To determine if the distribution of these changes differed significantly from zero, a Wilcoxon signed rank test was used throughout because several parameter changes were not normally distributed. Comparison between responders and nonresponders was performed using the Wilcoxon rank sum test.

Progression-free survival (PFS) was calculated from the on-study date until the date of progression, or date of removal from study without progres-

sion, as appropriate. OS was calculated from the on-study date until the date of death or last follow-up. The Kaplan-Meier method was used to determine the probability of PFS or OS as a function of time. Exact CIs were computed regarding the fraction of responders.

Correlations were performed using Spearman nonparametric correlation analysis. The correlation coefficient r should be interpreted as follows: $r > 0.70$ indicates a strong correlation; $0.5 < r < 0.7$ indicates moderate correlation; $0.3 < r < 0.5$ indicates weak to moderate correlation; $r < 0.30$ indicates a weak correlation. The P value associated with a correlation coefficient is for a test of whether $r = 0$, and as such, more emphasis in interpretation should be placed on the actual magnitude of the correlation coefficient.

In view of the large number of parameters explored in this study, we will consider P values such that $P < .01$ is to be associated with statistically significant effects, whereas those with $.01 < P < .05$ should be interpreted as exhibiting trends toward statistical significance. All P values are two-tailed and have not been formally adjusted for any multiple comparisons.

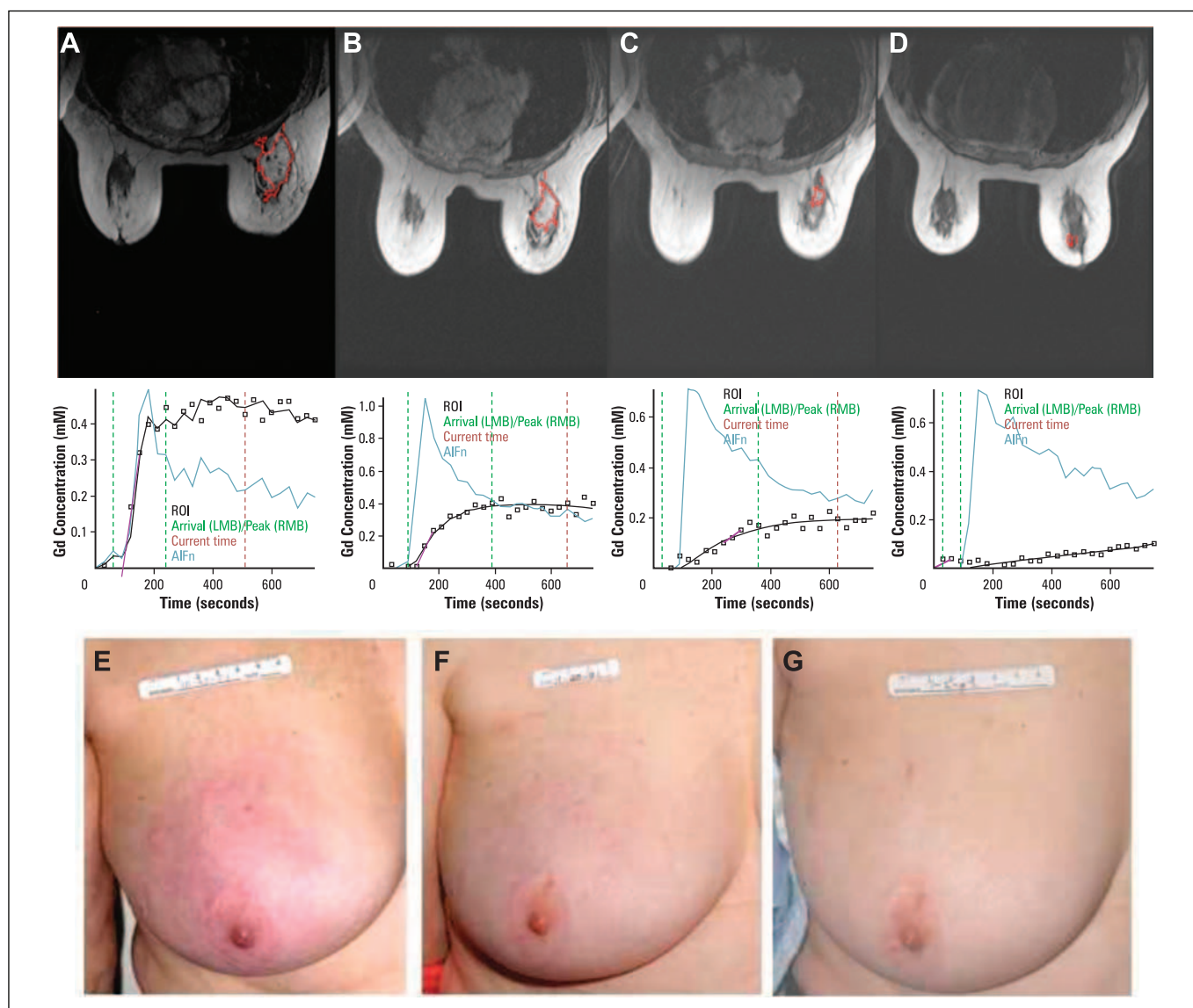


Fig 2. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and clinical responses in a patient with partial response. (A-D) DCE-MRI: tumor enhancement outlined in red on serial images, contrast kinetics (black line), and arterial input function (blue line) at (A) baseline, (B) post-cycle (C-) 1, (C) post-C4, and (D) post-C7. Clinical response at (E) baseline, (F) post-C1, and (G) post-C4.

Table 5. Change in Molecular Markers With Therapy

Marker	BL to C1				BL to C4/7				C1 to C4/7			
	No. of Patients*	Change (%)		P	No. of Patients	Change (%)		P	No. of Patients	Change (%)		P
		Median	Range			Median	Range			Median	Range	
Ki67	18	2.1	−68.61-07.3	.97	16	−35.1	−87.6-175.8	.025	16	−41.4	−88.2-355	.12
MVD	18	−14.6	−88.4-15,240	.97	16	16.6	−84.9-23,220	.82	16	−14.5	−79.2-215.9	.86
VEGF-A	18	−50	−100-11,900	.35	16	−44.9	−100-500	.80	15	0	−95-1,100	.63
p-VEGFR2 (Y996)	18	−69.2	−100-161	.025	16	−88.9	−100-512	.013	15	−49.5	−100-1.4	.081
p-VEGFR2 (Y951)	18	−66.7	−99.1-811	.004	16	−76.4	−99.8-901.7	.009	16	−26.1	−97.9-529.5	.86
VEGFR2	17	69.7	−93.9-16,560	.083	14	−28.3	−99.6-14,300	.70	14	−91.1	−99.7-14,300	.021
TUNEL	17	128.9	−93.1-2328.6	.0008	15	72.7	−77.6-927.3	.013	16	−13.6	−100-665	.64

NOTE. All values represent relative changes for the later time point compared with the earlier one indicated. *P* values are two tailed and were calculated using the Wilcoxon signed rank test.

Abbreviations: BL, baseline; C-, cycle; MVD, microvessel density; p-VEGFR2, phosphorylated VEGFR2; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

*Data missing in one patient at baseline and two patients post-C1.

RESULTS

Patient Characteristics

Between October 2001 and May 2004, 21 patients were enrolled onto this pilot study and followed through May 2, 2005. Baseline characteristics are summarized in Table 2. All patients received at least the first cycle of bevacizumab alone and are included in the analysis. Twenty patients had IBC and one patient had noninflammatory LABC (the study was briefly open to non-IBC patients).

Toxicity

All patients were assessable for toxicity assessment. Frequent and relevant toxicities are shown in Table 3. Eight patients had grade 3 hypertension with drug intervention required. Three patients had grade 3 hypertension at baseline and required an increase in drug therapy on study. One patient developed a catheter-related mural thrombus after nine cycles of therapy and was taken off study. Six patients had grade 1 bleeding: three gingival, two at the biopsy site, and one scleral hemorrhage. Five patients had wound-healing complications after surgery, including two with prolonged seromas, two with incisional separations (one requiring surgical repair), and one with prolonged time to closure of the primary wound.

Grade 2 asymptomatic decrease in LVEF was observed in two patients with right-sided breast cancer. In both patients, LVEF dysfunction occurred after all seven cycles of neoadjuvant therapy (total 300 mg/m² doxorubicin and 450 mg/m² docetaxel), surgery, and radiation therapy before starting C8. One patient was taken off study; her LVEF normalized 6 months after she discontinued therapy and received an angiotensin converting enzyme inhibitor. The second patient had normalization of her LVEF within 3 weeks and remained on study. Overall, there was a trend toward a significant decrease in the relative percentage change of LVEF after C7 (median, −6.2%; range, −19.1% to 8.2%; *P* = .013; *n* = 16; Fig 1). Other than these two patients, no patient had an absolute decrease in LVEF > 15% or a decrease in LVEF below the lower limit of normal. No segmental abnormalities were noted on echocardiogram.

Study Drug Administration

A total of 199 cycles of therapy were administered on this protocol as of May 2, 2005. The median relative dose-intensity for bevac-

zumab was 0.75 (range, 0.47 to 1.02), docetaxel was 1.00 (range, 0.79 to 1.02), and doxorubicin was 1.01 (range, 0.83 to 1.18). Sixteen patients completed all seven cycles of neoadjuvant therapy before surgery. Four patients required dose reductions while receiving bevacizumab and chemotherapy: two for grade 3 diarrhea and two for febrile neutropenia. One patient with febrile neutropenia required a second dose reduction for prolonged fatigue and sinusitis. Thirteen patients had surgery (12 mastectomy and one lumpectomy) and radiation therapy. The eight patients who did not have surgery had been taken off study during neoadjuvant therapy. Some of these patients went on to have surgery off study at a later time. Fifteen patients did not complete all 15 cycles for the following reasons: five, disease progression/recurrence; one, thrombus; one, asymptomatic decrease in LVEF; one, typhilitis; one, prolonged seroma; one, increased toxicity (hypertension, occipital neuralgia, carpal tunnel syndrome, plantar fasciitis); one, cholecystectomy; and four, optimal response not achieved.

Efficacy and Outcome

All 21 patients were assessed for response. On the basis of imaging modalities, 14 patients had a clinical partial response (cPR) representing an overall response rate (ORR) of 67% (95% CI, 43% to 85.4%). No complete responses were observed. Five patients had stable disease (SD) and 2 patients had progressive disease (PD). One of the patients with a cPR was unconfirmed (surgery was performed before confirmation). One patient (stage IIIB) with a cPR had a complete pathologic response (pCR). For purposes of analyses, patients were grouped into either responders (cPR) or nonresponders (SD + PD).

Median potential follow-up is 26.9 months. Median PFS was 25.3 months, with 1- and 2-year PFS probabilities of 77.5% and 53.3%, respectively. The 1-year overall survival probability was 90.5%, with 80.0% probability at 2 years. Median overall survival has not yet been reached.

DCE-MRI

Among the 21 patients, DCE-MRI was performed in 20 patients (19 IBC, one LABC) at baseline, 18 patients after C1, 19 patients after C4, and 15 patients after C7. In our implementation of DCE-MRI analysis, we evaluated three pharmacokinetic parameters in the modeling of the gadolinium contrast time passage through the tumor:

K^{trans} , which primarily reflects the wash-in of contrast agents; k_{ep} , which reflects the wash-out; and v_e , which represents the tumor over-all leakage space. As seen in Table 4, three parameters (K^{trans} , k_{ep} , v_e) representative of vasculature permeability and flow measured from the two compartment model were significantly decreased after C1. The decreases continued with the addition of cytotoxic chemotherapy. A greater change was observed from C1 to C4 than from C4 to C7, implying that the overall tumor rate of change in treatment effect occurred in the earlier course of therapy. Representative changes in DCE-MRI and corresponding clinical presentation in a patient with cPR are shown in Figure 2A and B. However, there were no significant differences in any of the DCE-MRI parameters from clinical responders to nonresponders.

Molecular End Points

To investigate whether bevacizumab has effects on tumor cells, p-VEGFR2 at tyrosine phosphorylation sites 951 and 996 (ie, activated VEGFR2 at Y951 and Y996) were examined. As shown in Table 5 and Figure 3A and C, a 66.7% median decrease compared with baseline in p-VEGFR2 (Y951) in tumor cells was observed with bevacizumab ($P = .004$); this decrease persisted with the addition of cytotoxic che-

motherapy (median decrease, 76.4% relative to baseline; $P = .009$). Similarly, p-VEGFR2 (Y996) was also decreased after bevacizumab ($P = .025$; Table 5; Fig 3A and D) and combination therapy ($P = .013$). In contrast to the decrease in p-VEGFR seen in patients with PR and SD, levels of p-VEGFR2 were high in both patients with PD. One of these patients had a substantial increase in p-VEGFR2 expression compared with baseline (Fig 3B). The other patient had the strongest expression in p-VEGFR2 of all patients after bevacizumab, although p-VEGFR2 expression could not be determined at baseline.

There was an increase in tumor apoptosis as measured by TUNEL assay (median increase, 128.9% relative to baseline; $P = .0008$; Table 5; Fig 4A and B); which again persisted with the addition of chemotherapy (72.7%; $P = .013$). As shown in Table 5, there was no significant change in tissue VEGF, MVD, VEGFR2 or tumor proliferation as measured with Ki67 with bevacizumab or combination therapy. In five paired biopsies at baseline and after C1, there was a trend toward a decrease in endothelial cell proliferation ($P = .063$).

VEGF expression decreased more in responders compared with nonresponders (57.6% [range, -100.0% to 11,900%] v 0% [range,

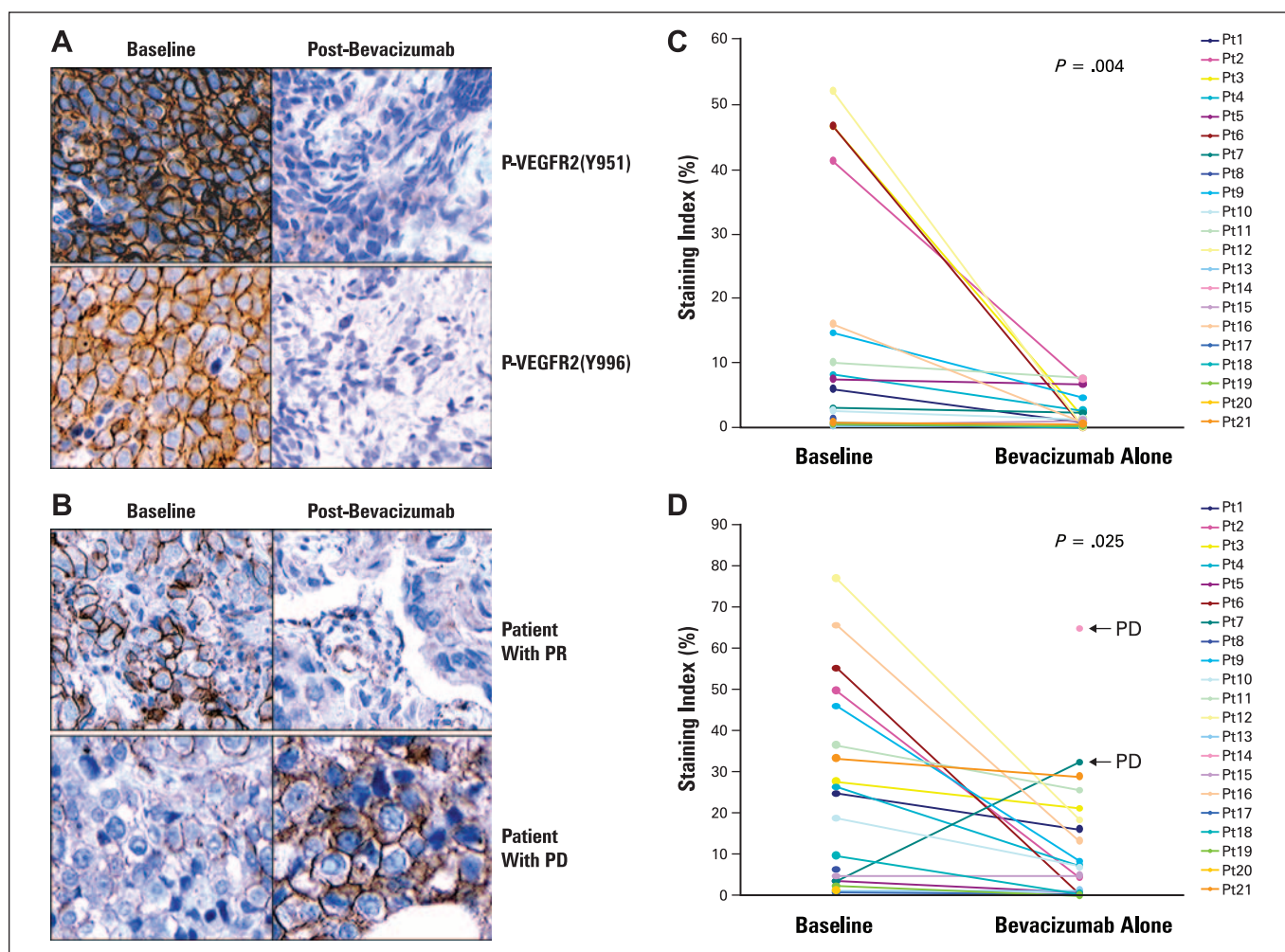


Fig 3. Phosphorylated vascular endothelial growth factor receptor 2 (p-VEGFR2) by immunohistochemistry. (A) p-VEGFR2 at Y951 and Y996 at baseline on left and post-cycle 1 on right. (B) p-VEGFR2 at Y951 in a patient (pt) with partial response (PR) on top and in a patient with progressive disease (PD) at bottom. (C) Changes in p-VEGFR2 (Y951) and (D) p-VEGFR2 (Y996) in all patients.

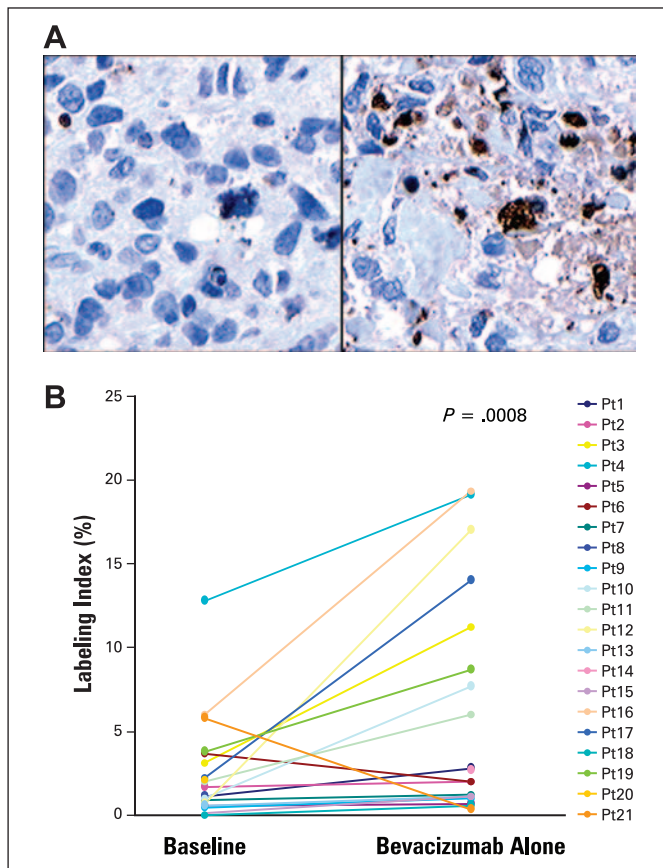


Fig 4. Tumor apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. (A) Tumor apoptosis at baseline (left) and post-cycle 1 (right). (B) Absolute change in tumor apoptosis in all patients. Pt, patient.

–50% to 420%]; $P = .037$) after C1. MVD, VEGFR2, p-VEGFR2, and TUNEL did not differ between the two groups. In examining the relationships of different markers at baseline, there was a strong correlation between the two VEGFR2 phosphorylation sites ($r = 0.85$; $P < .0001$). Moderate correlations were seen between p-VEGFR2 (Y996) and VEGFR2 ($r = 0.63$; $P = .0027$) and between MVD and VEGFR2 ($r = 0.57$; $P = .0093$). In evaluating C1 marker levels, a moderate inverse association was noted between K^{trans} and VEGFR2 ($r = -0.58$; $P = .018$).

DISCUSSION

We have demonstrated a significant decrease in VEGFR2 activation in tumor cells and increase in tumor apoptosis after one cycle of bevacizumab alone. VEGF released from tumor cells or inflammatory and endothelial cells is known to have multiple paracrine and autocrine effects. These effects have been demonstrated in preclinical settings in prostate cancer,¹⁹ head and neck cancers,²⁰ acute leukemias,²¹ and breast cancer.²² Expression of VEGFR2^{23–25} and p-VEGFR2^{26,27} have been reported previously in human breast cancer. However, this is the first clinical study to demonstrate that bevacizumab has a direct inhibitory effect on angiogenic parameters in tumor cells, possibly as a result of the disruption of both autocrine and paracrine functions of VEGF. Interestingly, endothelial proliferation was decreased in five of

five cases after bevacizumab, which also suggests an inhibitory effect on endothelium.

A striking membranous staining for p-VEGFR2 was found in the majority of pretreatment specimens. However, after bevacizumab, the staining was mostly cytoplasmic and had a marked decrease in staining intensity, suggesting the inactivation of the receptor. Consistent with the previous findings, staining in tumor VEGFR2 was cytoplasmic in all samples.²⁴ Dissimilar cellular localization of p-VEGFR2 and VEGFR2 are most likely a result of the difference in the activation state of the receptor with the activated receptor on the cell membrane and the inactivated in the cytoplasm.²⁸ Initially, we observed the decrease in p-VEGFR2 using anti-pY996, and the latter was confirmed with a second anti-p-VEGFR2 antibody at tyrosine phosphorylation site 951. These results suggest that two different phosphorylation sites of VEGFR2 representative of the activated receptor status are inhibited by bevacizumab.

The significant decrease in the receptor activation after bevacizumab was coupled with a marked increase in tumor apoptosis, but not changes in tumor proliferation. Possibly, anti-VEGF therapy with bevacizumab primarily affected survival pathways leading to induction of tumor apoptosis and did not have effects on cell cycle kinetics. Similar results have been seen in a study with neoadjuvant trastuzumab.²⁹ As would be expected with the addition of cytotoxic chemotherapy, there was a trend toward a significant decline in tumor proliferation after combination treatment with bevacizumab and chemotherapy ($P = .025$). Together, our data suggest that blockade of VEGF inhibits the activation of VEGFR2 and induces the tumor apoptosis. However, we could not formally compare the changes in patients with PR and SD to those of patients with progression (only two patients). Various doses of bevacizumab have been used in clinical trials, and it is possible that different doses could affect the molecular end points we evaluated.

Bevacizumab appeared to also have direct effects on tumor permeability and flow, as noted on DCE-MRI. Using the GKM two-compartment model, we found a significant decrease in K^{trans} , k_{ep} , and v_e after bevacizumab. The decreased vascularity may have contributed to the increase in tumor apoptosis.

Bevacizumab related toxicities were similar to those reported previously. Hypertension was medically manageable in all cases, left ventricular dysfunction was asymptomatic and recovered in both cases, and bleeding was mild in all cases. However, the incidence of delayed wound healing (24% of all patients and 39% of patients undergoing surgery) was higher than expected in comparison with previous studies.³⁰ In this study, bevacizumab was administered in the neoadjuvant setting, whereas in other trials, it was administered when extensive surgery was not usually required. Only six of the 21 patients were able to complete all 15 cycles of therapy. Nine patients could not complete therapy because of tumor recurrence or progression, or not achieving an optimal response, highlighting the poor prognosis in this patient population.

Clinical benefit with bevacizumab has been reported from randomized trials in metastatic colon, renal cell, and, most recently, breast cancer.^{31–33} A phase III trial in heavily pretreated breast cancer patients demonstrated a significant increase in ORR with the addition of bevacizumab to chemotherapy despite a lack of the improved PFS.³⁴ Results from a study with paclitaxel versus paclitaxel plus bevacizumab as first-line therapy in metastatic breast cancer showed a significant increase in ORR (14.2% v

28.2%; $P < .0001$) and PFS (6.11 v 10.97 months; $P < .001$) with the addition of bevacizumab.³³

In this pilot trial, we have demonstrated significant effects of anti-VEGF therapy on VEGFR2 activation, tumor apoptosis, and tu-

mor vascular permeability and flow as measured by DCE-MRI. This study has shed light on the establishment of **predictive markers** to bevacizumab treatment, although the findings described warrant further investigation in larger cohorts.

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GLOSSARY

Angiogenesis: The process involved in the generation of new blood vessels. While this is a normal process that naturally occurs and is controlled by “on” and “off” switches, blocking tumor angiogenesis (antiangiogenesis) disrupts the blood supply to tumors, thereby preventing tumor growth.

Apoptosis: Also called programmed cell death, it is a signaling pathway that leads to cellular suicide in an organized manner. Several factors and receptors are specific to the apoptotic pathway. The net result is that cells shrink, develop blebs on their surface, and their DNA undergoes fragmentation.

Bevacizumab: Also called Avastin, bevacizumab is a recombinant, humanized, monoclonal antibody that binds and neutralizes VEGF, thus acting as an antiangiogenic agent.

DCE-MRI (dynamic contrast-enhanced magnetic resonance imaging): A magnetic resonance imaging acquisition strategy involving multiple scans over a set volume during injection of a MR contrast agent.

Inflammatory breast cancer: Inflammatory breast cancer is a clinical diagnosis characterized by rapid enlargement of the breast, generalized induration in the presence or absence of a distinct breast mass, edema of the skin of the breast, erythema that must involve more than one third of the breast, and biopsy-proven carcinoma.

k_{ep} rate constant: Reverse rate constant from tumor to the vascular space determined on dynamic contrast-enhanced magnetic resonance imaging.

K^{trans} transfer constant: Forward transfer constant from vascular space to the tumor determined on dynamic contrast-enhanced magnetic resonance imaging.

p-VEGFR2 (phosphorylated vascular endothelial growth factor receptor 2): p-VEGFR-2 (also p-KDR) is the activated form of VEGFR2. VEGFR2 contains seven immunoglobulin-like loops extracellularly, and split tyrosine kinase domains in the intracellular domain. Upon binding of the ligand VEGF, VEGFR2 undergoes autophosphorylation on tyrosine residues located in the cytoplasmic part, and activates several signaling pathways that ultimately lead to angiogenesis and endothelial cell proliferation. Many tumors express VEGFR2 and the activated form, p-VEGFR2.

Predictive markers: Markers, biologic or molecular, that determine which treatment will increase the efficacy and improve outcome.

VEGF (vascular endothelial growth factor): VEGF is a cytokine that mediates numerous functions of endothelial cells including proliferation, migration, invasion, survival, and permeability. VEGF is also known as vascular permeability factor. VEGF naturally occurs as a glycoprotein and is critical for angiogenesis. Many tumors overexpress VEGF, which correlates to poor prognosis. VEGF-A, -B, -C, -D, and -E are members of the larger family of VEGF-related proteins.